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NepA is a structural cell wall protein involved in maintenance of spore dormancy in *Streptomyces coelicolor*

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Summary

Streptomyces have a complex morphogenetic programme culminating in the formation of aerial hyphae that develop into chains of spores. After spore dispersal, environmental signals trigger dormant spores to germinate to establish a new colony. We here compared whole genome expression of a wild-type colony of *Streptomyces coelicolor* forming aerial hyphae and spores with that of the *chp* null mutant that forms few aerial structures. This revealed that expression of 244 genes was significantly altered, among which genes known to be involved in development. One of the genes that was no longer expressed in the Δ *chpAB-CDEFGH* mutant was *nepA*, which was previously shown to be expressed in a compartment connecting the substrate mycelium with the sporulating parts of the aerial mycelium. We here show that expression is also detected in developing spore chains, where NepA is secreted to end up as a highly insoluble protein in the cell wall. Germination of spores of a *nepA* deletion mutant was faster and more synchronous, resulting in colonies with an accelerated morphogenetic programme. Crucially, spores of the Δ *nepA* mutant also germinated in water, unlike those of the wild-type strain. Taken together, NepA is the

first bacterial structural cell wall protein that is important for maintenance of spore dormancy under unfavourable environmental conditions.

Introduction

The filamentous bacterium *Streptomyces coelicolor* is a multicellular organism that undergoes a complex programme of morphological development (for reviews see Claessen *et al.*, 2006; Elliot *et al.*, 2008). After spore germination a vegetative mycelium is formed, which consists of hyphae that colonize the substrate. The substrate mycelium is subject to complex differentiation processes that are accompanied by a phase of massive cell death (Wildermuth, 1970; Manteca *et al.*, 2005; 2006; 2007). During this phase, vegetative hyphae are compartmentalized into multiple segments, some of which disintegrate, while others proliferate to give rise to a second vegetative mycelium. At the onset of second vegetative mycelium formation, hyphae start to grow out of the substrate to produce aerial hyphae (Manteca *et al.*, 2005; 2006; 2007). The apical part of these aerial hyphae develops into a chain of spores, whereas the role of the non-sporulating part, called 'the subapical stem' (Dalton *et al.*, 2007), has not yet been elucidated.

The mechanics of aerial hyphae formation is relatively well understood. Surfactants are secreted into the aqueous environment enabling hyphae to breach the water surface tension and to grow into the air. One of these surfactants, perhaps produced by the second vegetative mycelium, is the small lantibiotic-like peptide SapB (Willey *et al.*, 1991; Tillotson *et al.*, 1998; Kodani *et al.*, 2004). Members of another class of proteins, called the chaplins, were shown to have similar surface tension-reducing properties (Claessen *et al.*, 2003; Elliot *et al.*, 2003). *S. coelicolor* has eight chaplin proteins (ChpA–H) of which two, ChpE and ChpH, are secreted into the medium during vegetative growth (D. Claessen, unpubl. results). As such they fulfil a function similar to that of SapB (Claessen *et al.*, 2003; Capstick *et al.*, 2007). During the emergence of aerial hyphae, ChpA–H are secreted and assemble on the outer surface into an hydrophobic layer composed of 4- to 6-nm-wide fibrils. The rodlin proteins RdlA and RdlB arrange the chaplin fibrils into a characteristic mosaic of pairwise aligned, 8- to

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12-nm-wide fibres, which is known as the rodlet layer (Claessen *et al.*, 2002; 2004). This rodlet layer has an amyloid-like nature and as such is highly insoluble (Claessen *et al.*, 2002; 2003; 2004; Gebbink *et al.*, 2005). Both the rodlin and the chaplin remain associated with the cell wall when treated with hot 2% SDS and can only be dissociated with trifluoroacetic acid (TFA).

The *bld* genes control the onset of aerial hyphae formation by regulating the expression of the genes involved in the production of SapB (Willey *et al.*, 1991; 1993; Nguyen *et al.*, 2002; Kodani *et al.*, 2004), the rodlin (Claessen *et al.*, 2002) and the chaplin (Claessen *et al.*, 2003; Elliot *et al.*, 2003). *bld* mutants that fail to form aerial hyphae and spores on rich media also do not produce the structural proteins involved in their formation. Interestingly, expression of the rodlin genes was also reduced in the Δ *chpABCDEH* mutant (in which *chpF* and *chpG* are still present) that is severely affected in aerial growth despite the presence of an intact *bld* cascade. Notably, the few aerial hyphae that were formed by the Δ *chpABCDEH* mutant expressed the rodlin genes at the same level as those of the wild-type strain (Claessen *et al.*, 2004). Taken together, it was proposed that expression of rodlin and possibly other developmental genes requires additional regulation, which was termed the *sky* pathway (Claessen *et al.*, 2004; 2006). This regulatory mechanism would only be activated when hyphae start to grow into the air (Claessen *et al.*, 2004; 2006).

Expression of *sigN* also appears to depend on the formation of aerial hyphae (Dalton *et al.*, 2007). This gene encodes a sigma factor required for transcription of genes in the subapical stem. One of the targets of SigN is a gene encoding a small peptide designated NepA (Dalton *et al.*, 2007). We here show that *nepA* is a *sky* pathway target that is not only expressed in the subapical stem but also in developing spore chains. Here, NepA is secreted to end up as a highly insoluble cell wall protein that functions in maintenance of spore dormancy.

Results

Global transcriptome analysis of the Δ *chpABCDEFGH* mutant

We previously showed that expression of the rodlin genes was reduced in chaplin mutant strains that are structurally impaired in aerial growth (Claessen *et al.*, 2004). To study the effect of the absence of chaplins on global gene expression, DNA microarrays were hybridized with RNA isolated from wild-type colonies in several stages of development and with RNA from the isogenic Δ *chpABCDEFGH* mutant that scarcely produced aerial structures (see *Experimental procedures* for details). Array data were confirmed with Northern analysis using probes for

genes *rdlA*, *rdlB* (Claessen *et al.*, 2004), *SCO4173* (data not shown) and *nepA* (Dalton *et al.*, 2007; see below, Fig. 2). One hundred genes were identified whose transcription was decreased (or absent) in the Δ *chpABCDEFGH* mutant (Fig. 1A; Table S1), while the expression of 144 genes was upregulated (Fig. 1B and Table S2) as was determined by using Rank Product analysis (Breitling *et al.*, 2004; Hesketh *et al.*, 2007). Interestingly, 17 of the 100 downregulated genes were also found to be expressed at a lower level in a *bldA* mutant (Hesketh *et al.*, 2007). Among the other genes that were no longer expressed or downregulated in the *chp* null mutant, 23 encode secreted proteins, including *rdlB*, *chpA*, *chpE* and *chpF* [serving as an internal control; the other *chp* genes, as well as *rdlA* and *ramS*, were either not spotted (due to failure to design suitable specific PCR primers) or failed to pass the quality control during filtering, but were silent or downregulated upon manual inspection], four encode transcriptional regulators and 35 encode hypothetical proteins (Table S1). Remarkably, several genes found to be upregulated in the Δ *chpABCDEFGH* mutant are involved in primary metabolism, such as glyceraldehyde-3-phosphate dehydrogenase (*gap1*), pyruvate dehydrogenase (*aceE1*) and fumarate hydratase (*fumB*) (Table S2). Notably, none of the classical *bld* genes, except for *bldKB* (*SCO5113*) and *bldKC* (*SCO5114*) of the *bldK* locus (Nodwell *et al.*, 1996), nor any *whi* genes were differentially expressed in the Δ *chpABCDEFGH* mutant.

nepA (Dalton *et al.*, 2007) was identified as one of the most highly expressed genes during formation of aerial hyphae in the wild-type strain. Interestingly, expression of this gene was strongly reduced in the Δ *chpABCDEFGH* mutant (Fig. 1A). Gene *nepA* was predicted, like rodlin and chaplin, to encode a small secreted hydrophobic polypeptide, making it an interesting target for further characterization. It encodes a protein of 108 amino acids of which the first 29 amino acid residues are predicted to be the signal sequence (Dalton *et al.*, 2007). Cleavage of this signal peptide during secretion results in a mature protein of 79 amino acids with a predicted molecular weight of 7725 Da. Hydropathy analysis reveals a pattern of alternating hydrophobic-hydrophilic regions. The presence of several acidic amino acids results in an isoelectric point (pI) of 3.12. Homologues of *nepA* are exclusively found in *Streptomyces* genome sequences. The N-terminal region of mature NepA appears to be the least conserved and is even absent in the *Streptomyces avermitilis* SAV4213 homologue (Dalton *et al.*, 2007).

nepA is expressed in aerial hyphae and spores

To study the expression pattern of *nepA* in more detail, a probe directed against the coding sequence of this gene was hybridized to RNA from cultures at various stages of

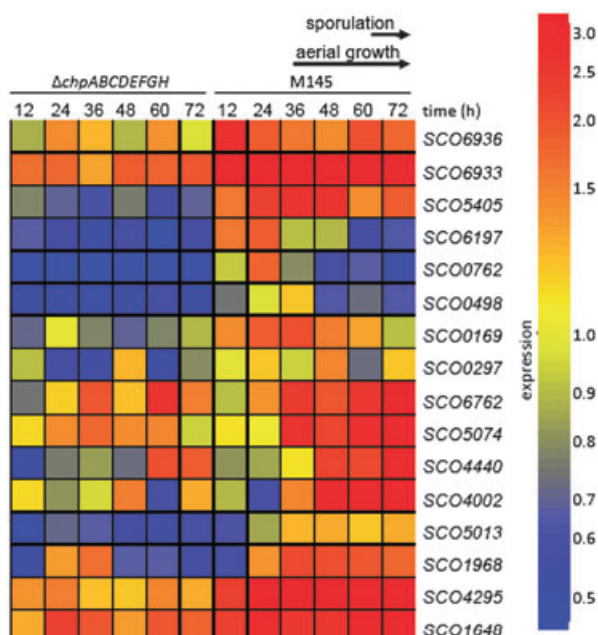
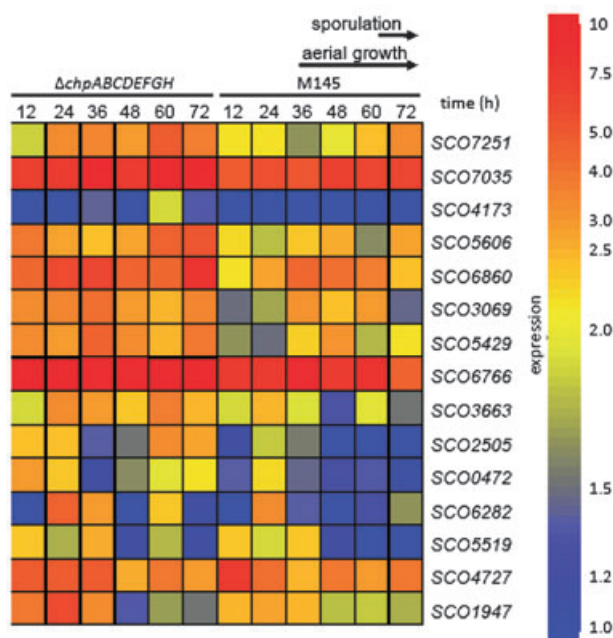
A Down-regulated in the *chp* null mutant**B** Up-regulated in the *chp* null mutant

Fig. 1. DNA microarray-based expression analysis during development of the wild-type strain M145 and the isogenic $\Delta chpABCDEFGH$ mutant. Sixteen (A) and 15 (B) genes were significantly down- and upregulated, respectively, in the $\Delta chpABCDEFGH$ strain with a pff value (probability of false prediction) of 0 derived from Rank Products analysis of triplicate data sets. All genes with a pff of < 0.1 are shown in Tables S1 and S2.

development. *nepA* mRNA started to accumulate in 36-h-old cultures that initiated aerial hyphae formation. Expression of *nepA* mRNA increased strongly between 36 and 48 h, and remained high at least until 72 h of growth, coinciding with sporulation (Fig. 2A). In agreement with the microarray analysis, no *nepA* mRNA accumulated in the $\Delta chpABCDEFGH$ mutant (Fig. 2B).

Previously, *nepA* expression was localized in the subapical stem, which represents the non-sporulating part of aerial hyphae (Dalton *et al.*, 2007). The presence of *nepA* mRNA transcripts during sporulation suggested that *nepA* might have a role not only in the subapical stem but also in spores. To spatially localize expression of *nepA* during development, *S. coelicolor* M145 was transformed with vector pIJ8630-*nepA* encompassing the promoter region of *nepA* cloned in front of the *eGFP* gene. No GFP fluorescence was detected in vegetative mycelium in cross-sections of colonies of this recombinant strain (Fig. 3A and B). In contrast, GFP localized specifically in the upper zone corresponding to the region where aerial mycelium was being formed (Fig. 3A and B). Notably, *nepA* expression was weak in young aerial hyphae (Fig. 3E and F) but was at least fivefold stronger in older aerial hyphae that had started to sporulate (Fig. 3E–H). Taken together, these results show that *nepA* is not exclusively expressed in the subapical stem (Dalton *et al.*, 2007), but is also highly active in developing spore chains.

Deletion of *nepA* results in accelerated spore germination and development

The coding sequence of *nepA* was replaced in *S. coelicolor* M145 by the apramycin resistance cassette

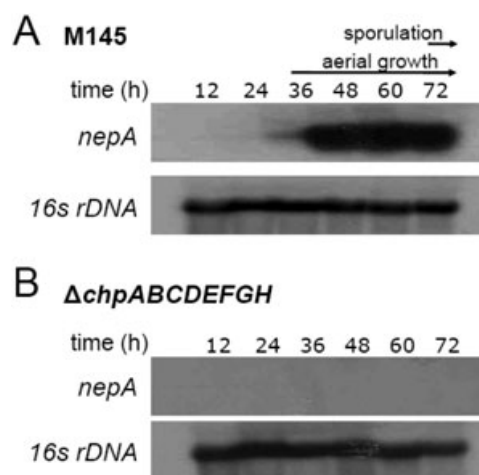


Fig. 2. Temporal expression of *nepA* in *S. coelicolor* grown on solid R5 medium. *NepA* transcript was detected in the wild-type strain during the formation of aerial hyphae and spores (A), whereas the $\Delta chpABCDEFGH$ mutant failed to accumulate *nepA* transcript (B). Hybridization with 16s rDNA served as a loading control.

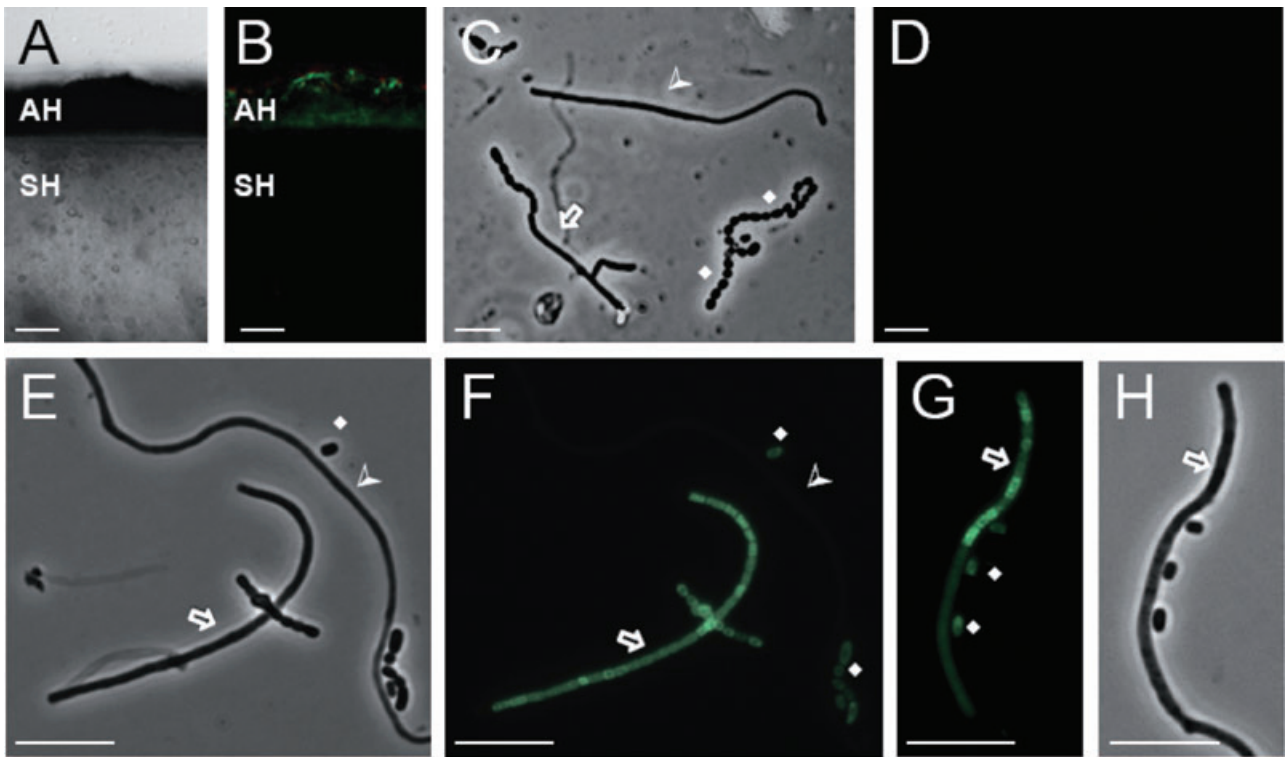


Fig. 3. Spatial expression of *nepA* during development of *S. coelicolor* wild type visualized using eGFP as a reporter. GFP expression was observed in the upper zone of the colony corresponding to the aerial hyphae (AH; A and B). Substrate hyphae (SH; A and B) were not fluorescent. Analysis at higher magnification (C–H) showed that GFP expression was weak in young aerial hyphae (\blacktriangleleft) (E and F) and high in mature spores (\blacklozenge) and in aerial hyphae that initiated sporulation (\blacktriangleleft) (E–H). Microscope settings and exposure time were adjusted such that the wild-type strain carrying the empty plasmid pIJ8630 was not fluorescent (C and D). (A), (C), (E), (H) represent bright-field images, those of (B), (D), (F), (G) were obtained with fluorescence microscopy. Bars represent 50 μ m in (A) and (B) and 10 μ m in (C)–(H).

using the Redirect procedure (see *Experimental procedures* for details). Inactivation of *nepA* was confirmed by PCR and Southern analysis (Fig. S1A and B) in three mutants obtained from two independent conjugation events. As these mutants showed a similar phenotype, one was taken for further analysis and designated as the Δ *nepA* mutant. Spore germination started earlier in the Δ *nepA* mutant when spores were inoculated on solid MS medium (Fig. 4). Only 6% of the wild-type spores had germinated 3 h after inoculation. This number was at least three times higher (19%) in the Δ *nepA* mutant. The number of germinating spores of the Δ *nepA* mutant rapidly increased and after 6 h 88% of the Δ *nepA* spores had germinated, in contrast to only 58% of the wild-type spores (Fig. 4B and C). After 10 (Δ *nepA* mutant) and 14 h (wild-type strain) close to 100% of the spores had formed germ tubes (Fig. 4). Thus, within 4 h (between 2 and 6 h after inoculation) the vast majority of the spores of the Δ *nepA* mutant had germinated whereas this took twice as long (between 2 and 10 h after inoculation) in the wild-type strain. This shows that germination started not only earlier but was also more synchronized in the Δ *nepA* mutant (Fig. 4A). The number of germ tubes produced per

spore was similar in the Δ *nepA* mutant and the wild-type strain (data not shown). In addition, spores were equally able to withstand heat, detergent, lysozyme and sonication (data not shown).

Formation of vegetative mycelium in the Δ *nepA* strain was phenotypically indistinguishable from that of the wild type. Both strains eventually developed an alternating pattern of live and dead segments (data not shown). Interestingly, proliferation of the second vegetative mycelium from the viable segments occurred synchronously in the Δ *nepA* mutant after 10–13 h (Fig. 5B). In contrast, formation of the second vegetative mycelium in the wild-type strain started locally (Fig. 5A) and eventually covered the whole plate after 14–18 h (data not shown). Aerial mycelium formation was also accelerated in the Δ *nepA* mutant and was macroscopically visible after 20–24 h of growth on solid MS medium, whereas the wild-type strain started to form aerial hyphae after 26–30 h of growth (Fig. 5C). Initially, aerial hyphae of the wild type erected individually from the substrate, whereas those of the Δ *nepA* strain frequently formed bundles (Fig. 5E and F respectively). These bundled hyphae formed a dense aerial mycelium, producing morphologically normal chains of spores. In

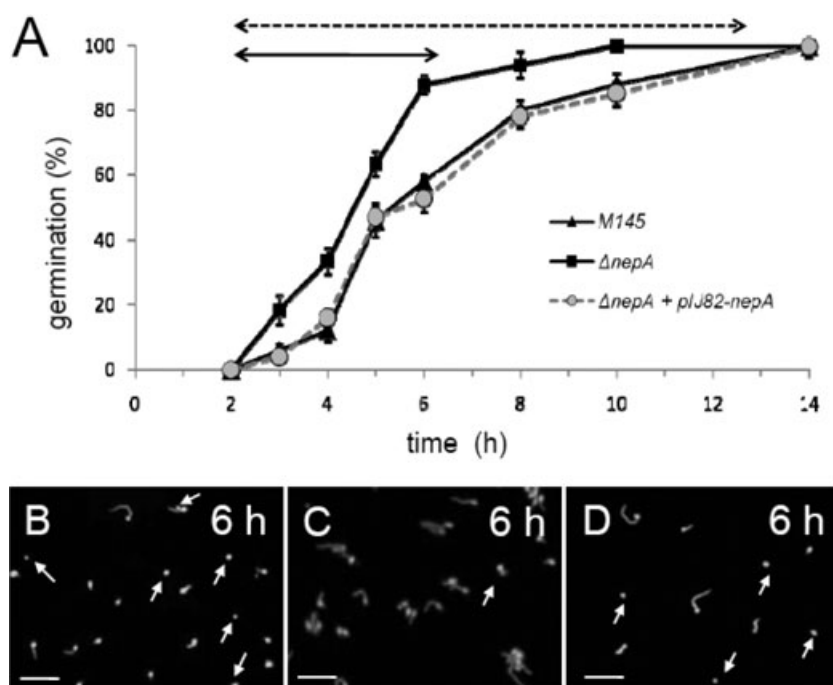


Fig. 4. Statistical analysis of spore germination in the wild-type strain M145, the $\Delta nepA$ mutant and the complemented $\Delta nepA$ mutant on solid MS medium (A). Analysis of spores of the wild type (B), the $\Delta nepA$ mutant (C) and the complemented $\Delta nepA$ mutant (D) 6 h after inoculation shows that spore germination is faster in the mutant strain. (A) Arrows indicate the time frame in which 90% of the spores had germinated. The solid arrow represents the $\Delta nepA$ mutant, whereas the dashed arrow represents the wild type and the complemented $\Delta nepA$ mutant. Arrows (B–D) point at spores that have not yet germinated. Bars indicate 10 μm .

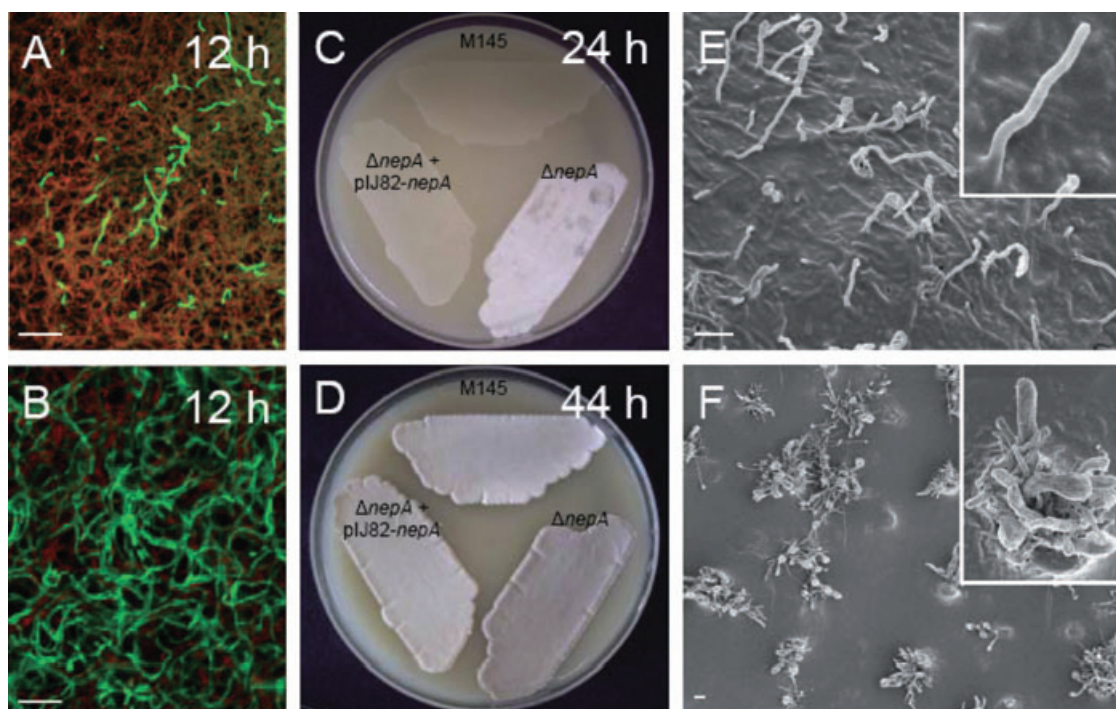


Fig. 5. Phenotypic analysis of the developmental cycle of the *S. coelicolor* $\Delta nepA$ mutant on solid MS medium analysed by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). After 12 h of growth, the second vegetative mycelium (coloured green resulting from SYTO 9 staining) arose less synchronously in the wild-type strain (A) as compared with the $\Delta nepA$ mutant (B). In addition, aerial hyphae formation (C) and sporulation (D) were earlier in the $\Delta nepA$ mutant compared with the parental wild-type strain M145 and the complemented $\Delta nepA$ strain. Aerial hyphae of the wild type (E) grew individually into the air, whereas those of the $\Delta nepA$ mutant frequently formed bundles (F).

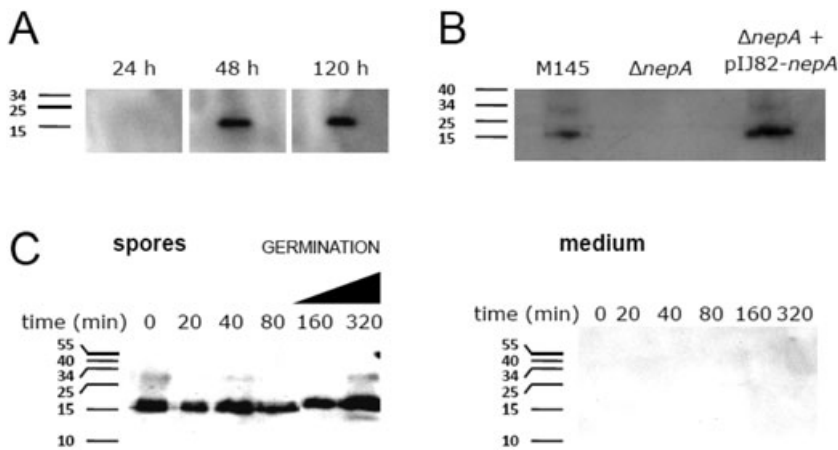


Fig. 6. Localization of NepA in *S. coelicolor* determined by Western analysis.

A. NepA was detected in TFA extracts of cell walls of 48- and 120-h-old cultures of the wild-type strain that had formed aerial hyphae and spores respectively. In contrast, NepA was not observed in 24-h-old cultures forming substrate mycelium only.

B. NepA was also detected in TFA extracts of cell walls of purified spores of the wild-type strain and the complemented $\Delta nepA$ mutant, but not in spores of the $\Delta nepA$ mutant.

C. NepA remains present in the spore cell wall during germination as an SDS-insoluble but TFA-extractable protein and is not released in the extracellular environment.

addition to early aerial growth, sporulation also started much earlier in the $\Delta nepA$ mutant (Fig. 5D). The mutant strain had formed 25–50 times more spores after 44 h of growth on MS medium. However, eventually the total number of spores produced by the wild type and the $\Delta nepA$ strain was the same (data not shown). The developmental acceleration of the $\Delta nepA$ strain was also observed on SF-glucose medium and R5 medium but was most prominent on minimal medium with mannitol or glucose as the carbon source. Introduction of plasmid pIJ82-*nepA* carrying the *nepA* gene complemented the altered germination dynamics of the $\Delta nepA$ mutant (Fig. 4A and D) and restored development such that it was comparable to the wild-type strain (Fig. 5C and D). In summary, these data show that inactivation of the *nepA* gene leads to early and synchronized spore germination and an accelerated developmental programme.

NepA is a highly insoluble cell wall protein of aerial hyphae and spores

The production of NepA in aerial hyphae and developing spore chains, and the predicted presence of a Sec-dependent secretion signal prompted us to investigate the possibility that NepA was targeted to the cell wall. To this end, cell extracts of mycelium that had been grown on MS medium for 24, 48 and 120 h were analysed for the presence of NepA using polyclonal antibodies raised against a mixture of NepA-derived peptides. No NepA-specific signal was detected in total cell extracts of the cultures that had been taken up directly in SDS sample buffer (data not shown). However, a strong signal was detected when SDS-treated cell walls of 48- and 120-h-old cultures were extracted with TFA. The apparent molecular weight of 16 kDa is twice as large as its predicted molecular weight of 7725 Da (see above), perhaps because NepA forms a dimer that is not dissociated by

SDS and TFA. This signal was absent in cell wall extracts from 24-h-old mycelium (Fig. 6A). The immuno-signal was also absent in a TFA extract of purified spores of the $\Delta nepA$ mutant, whereas in TFA extracts of wild-type spores as well as spores of the complemented *nepA* mutant strain, NepA was clearly detected (Fig. 6B). Immuno-labelling also showed the presence of NepA in cell walls of the sporulation-deficient mutants *whiA* and *whiH*, the $\Delta rdlAB$ mutant, the $\Delta chpABCDEH$ mutant (in which *chpF* and *chpG* are still present) and the $\Delta chpAB-CDEFGH$ mutant (Fig. S2 and data not shown). In case of the *chp* mutants, the amount of NepA was much lower compared with the other strains. It correlated with the number of aerial hyphae that were formed and the amount of the 'aerial hyphae-specific' rodlin proteins (Fig. S2). In addition, introduction of plasmid pIJ8630-*nepA* into the $\Delta chpABCDEH$ mutant showed that the level of GFP expression per aerial hypha was similar in the $\Delta chpABCDEH$ mutant and the wild-type strain (data not shown), as was shown before for the rodlin (Claessen *et al.*, 2004). These results show that NepA is secreted by aerial structures and ends up in the cell wall where it is present as an SDS-insoluble but TFA-extractable protein.

The presence of NepA in the cell wall of spores and the phenotype of early spore germination in the $\Delta nepA$ mutant prompted us to assess whether NepA is released into the medium during spore germination. Western analysis revealed that NepA remained associated with the cell wall in an SDS-insoluble but TFA-extractable form and was not released in the medium up to 320 min after the heat shock that induced germination (Fig. 6C). The fact that similar amounts of NepA could be extracted throughout this time period shows that NepA is a structural component of the spore cell wall with similar solubility properties as the rodlin and chaplin (Claessen *et al.*, 2002; 2003).

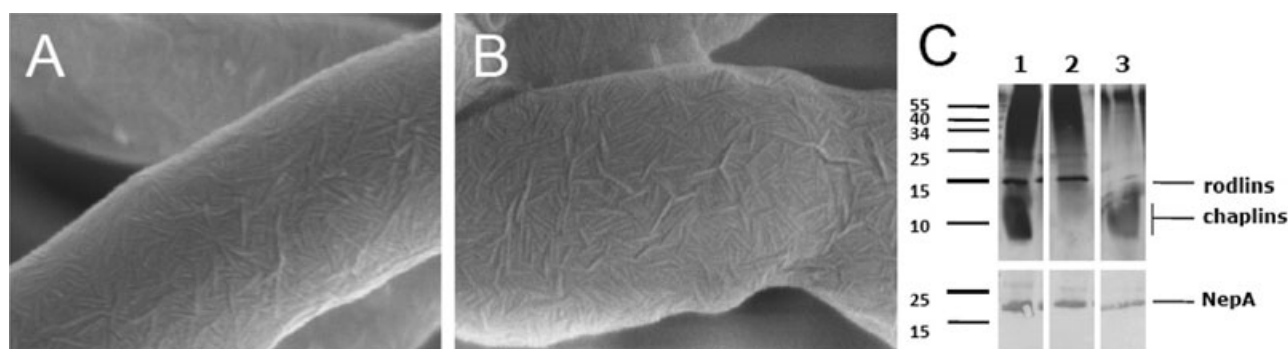


Fig. 7. NepA assembles into an SDS-insoluble structure but does not affect the formation of the rodlet layer.

A and B. Rodlets were observed by scanning electron microscopy both in the wild type (A) and in the *nepA* mutant (B).

C. SDS-PAGE (top) and Western analysis (bottom) of a TFA extract of wild-type cell walls. Analysis of a TFA extract (lane 1) revealed the presence of the rodlin and chaplin (which run as a smear and not as discrete bands) by silver staining after SDS-PAGE (top) and NepA by immuno-detection after Western blotting (bottom). A large air–water interface introduced by vortexing the water-solubilized TFA extract resulted in aggregation of the chaplins and a substantial amount of NepA (lane 3; note that aggregated proteins were separated from the soluble proteins by centrifugation, after which the pelleted proteins were dissociated with TFA before being subjected to SDS-PAGE), whereas the rodlin and the remaining part of NepA remained present in the supernatant (lane 2).

NepA does not affect formation of the rodlet layer

Since NepA, like rodlin and chaplin, resides in the cell wall in an SDS-insoluble but TFA-extractable protein, it was hypothesized that NepA is involved in formation of the rodlet layer. Analysis of the $\Delta nepA$ mutant, however, revealed the presence of the characteristic rodlet layer on the surface of aerial hyphae and spores, indistinguishable from the wild-type strain (Fig. 7A and B). Notably, germination of the $\Delta rdlAB$ mutant, the $\Delta chpABCDEH$ mutant and the $\Delta chpABCDEFGH$ mutant was similar to that in the wild type, indicating that defects in the rodlet layer do not affect germination (data not shown).

To investigate whether NepA, rodlin and chaplin interact, aqueous solutions of TFA extracts of cell walls were vortexed (thereby introducing a large air–water interface) and centrifuged. The supernatant was analysed by SDS-PAGE and Western analysis directly, whereas the pellets were taken up in SDS sample buffer after a TFA treatment. The chaplins and about 50% of NepA were found in the pellet fraction, whereas the rodlin was found in the soluble fraction (Fig. 7C). In contrast to the assembled chaplins, precipitated NepA was found to be largely SDS-soluble, irrespective of a TFA treatment. This indicates that TFA extraction may have affected the native NepA structure (data not shown).

NepA is involved in spore dormancy

To assess the role of NepA in spore germination, we tested spores for their ability to germinate in the absence of nutrients. Spores of the $\Delta nepA$ strain readily started to germinate in water after 2–3 days (Fig. 8B). In contrast, spores of the wild type (Fig. 8A) and the

complemented $\Delta nepA$ mutant (Fig. 8C) did not germinate under this condition. Longer incubation of these spores in water resulted in formation of large spore clumps in the $\Delta nepA$ mutant (Fig. 8E), which were limited in size and number in the wild type (Fig. 8D) and the complemented $\Delta nepA$ mutant (Fig. 8F). During longer incubation in water, cell lysis was frequently observed in the $\Delta nepA$ mutant but this was also observed to a limited extent in the wild type and the complemented $\Delta nepA$ mutant (data not shown). Similar results were obtained with strain K106 ($\Delta nepA$, Dalton *et al.*, 2007). No significant differences in swelling of germinating spores were detected between the strains that did or did not produce NepA (data not shown).

To test spore germination under hypertonic conditions, spores were suspended in 250 mM NaCl or 20% glycerol (v/v). Spores of the $\Delta nepA$ mutant germinated similarly in water and 20% glycerol. The presence of glycerol also did not affect the germination behaviour of the spores of the wild type and the complemented mutant, which scarcely germinated under these conditions (data not shown). Spores of all strains clumped under high-salt conditions, hampering quantitative analysis of spore germination. Zeta potential distribution measurements were used to determine the overall surface charge of spores. This was done to assess whether spore clumping could be caused by neutralization of the surface charge by salt. Indeed, both wild-type and $\Delta nepA$ mutant spores were negatively charged at physiological pH (i.e. pH 7) and remained so until the pH became as low as 3 (Fig. S3). In summary, the presence of *nepA* prevents spore germination in the absence of nutrients, revealing that NepA is involved in maintenance of spore dormancy under unfavourable conditions.

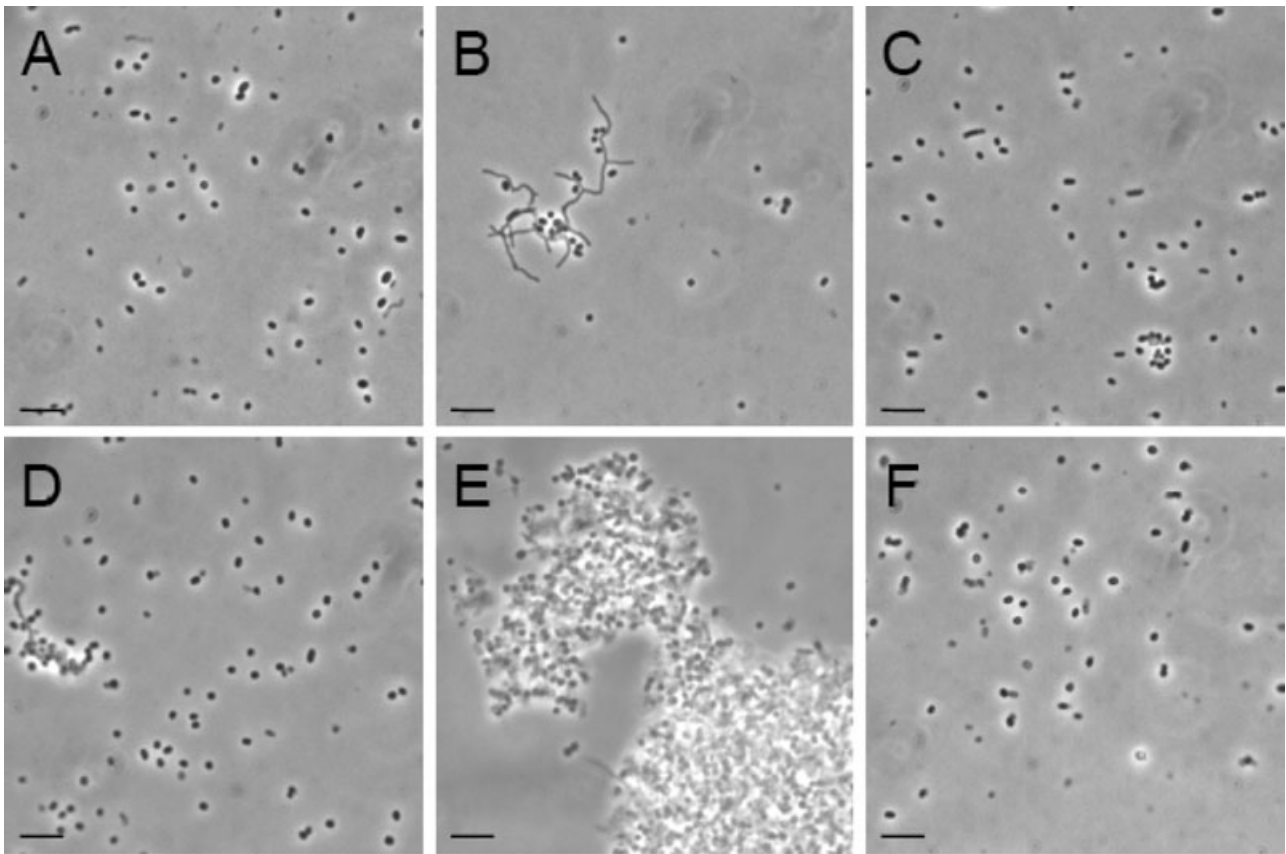


Fig. 8. Spores of the $\Delta nepA$ mutant germinate after 2 days in water (B), in contrast to the wild type (A) and the complemented $\Delta nepA$ mutant (C). After 7 days of incubation, large clumps of germinated spores are visible in the $\Delta nepA$ mutant (E), which were occasionally present in the wild type (D) and the complemented $\Delta nepA$ mutant (F). Bars represent 10 μm .

Discussion

The nepA gene is a novel target of the sky pathway

Streptomyces undergo complex morphological changes during their life cycle. Although formation of aerial hyphae and spores have been studied well, very little is known about control of spore dormancy and germination. Here we report that the *nepA* gene (Dalton *et al.*, 2007) encodes a structural cell wall protein that is involved in maintenance of spore dormancy in *S. coelicolor*. To our knowledge, this is the first example of such a protein identified in the bacterial domain tailored for this process.

It was shown previously that expression of the rodlin genes *rdlA* and *rdlB* was low in colonies of the $\Delta chpABCDEH$ mutant and even more reduced in the $\Delta chpABCDEFH$ strain. Both strains are severely affected in their formation of aerial hyphae. Interestingly, *rdl* expression within individual aerial hyphae was not affected in the mutant strains (Claessen *et al.*, 2004). From this it was concluded that the *rdl* genes are only expressed once a hypha senses that it had started to grow into the air

(Claessen *et al.*, 2006). This regulatory mechanism, termed the *sky* pathway (Claessen *et al.*, 2006), would thus act downstream of the *bld* genes. In a whole genome expression analysis, expression of 244 genes was found to be changed significantly in the $\Delta chpABCDEFGH$ strain compared with the wild type. Among the genes identified, there was a significant overlap with genes differentially expressed in a *bldA* mutant that was grown as a liquid shaken culture (Hesketh *et al.*, 2007). Apparently, these genes that are normally expressed in vegetative hyphae are under control of *bldA* as well as the *sky* pathway. In addition, expression of all previously known genes encoding structural proteins involved in morphogenesis, i.e. *rdlA*, *rdlB*, *ramS* (encoding the SapB precursor; Kodani *et al.*, 2004), were found to have reduced expression. In contrast, genes involved in the primary metabolism were significantly higher expressed in the $\Delta chpABCDEFGH$ strain, suggesting that the *sky* pathway plays a role in metabolic change during development. Future research will be devoted to unravel the mechanism of regulation of the *sky* pathway.

One of the genes that was downregulated in the $\Delta chpABCDEFGH$ strain, and also in a *bldN* mutant (Elliot

et al., 2003), was *nepA*. This gene was previously shown to be under control of the RNA polymerase sigma factor SigN and was found to be expressed in the sub-apical stem of aerial hyphae (Dalton *et al.*, 2007). This stem is believed to connect the aerial tip compartment and the vegetative mycelium of the *Streptomyces* colony. Using GFP as a reporter, we here showed that *nepA* is expressed in the whole filament of mature, but not young, aerial hyphae. Expression was also observed in developing spore chains. This localization agreed with a Northern analyses showing that the *nepA* transcript was abundantly present during aerial hyphae formation and sporulation. The presence of NepA in both aerial hyphae and spores supported the expression analysis. The temporal and spatial expression of *nepA* explains why its expression was not detected in colonies of the Δ *chpABCDEFGH* strain as this strain forms only a small number of aerial hyphae and spores. However, GFP reporter studies and protein extraction revealed that expression of *nepA* per aerial hypha was not affected. A similar phenomenon was observed with the rodlines (Claessen *et al.*, 2004; 2006).

Absence of the structural protein NepA results in accelerated spore germination

NepA was found to be an SDS-insoluble but TFA-extractable protein after its deposition in the cell wall. The rodlines and chaplins that form the rodlet layer in *S. coelicolor* have similar solubility characteristics (Claessen *et al.*, 2002; 2003). However, NepA does not have a role in formation of the rodlet layer since rodlets of the wild type and the Δ *nepA* strain were indistinguishable.

Spores of the Δ *nepA* mutant were different with respect to their germination behaviour. In minimal or complex medium spores of the mutant strain germinated faster and more synchronous. We propose that the accelerated spore germination explains why the Δ *nepA* mutant also showed faster and synchronized formation of the second mycelium and of aerial hyphae and spores. The synchronization may also explain why aerial hyphae of the *nepA* mutant strain bundle. Bundling would be the result of a larger number of hyphae that escape into the air at a certain time point and position.

Dalton *et al.* (2007) reported a conditionally bald phenotype when the Δ *nepA* mutant was grown on minimal medium with glucose as the carbon source. At present we have no explanation for the differences in the phenotypes in our study and that of Dalton *et al.* (2007). At least, in our hands the phenotypes of the Δ *nepA* mutant could be rescued by introduction of a wild-type copy of the *nepA* gene.

NepA affects spore dormancy

The molecular mechanism that controls spore germination in *Streptomyces* is poorly understood. Early studies report that dormant spores initially darken, which requires divalent metal ions, like Ca^{2+} , Mg^{2+} or Fe^{2+} (Hardisson *et al.*, 1978). Next, spores start to swell as a consequence of the influx of water, followed by the emergence of a germ tube (Hardisson *et al.*, 1978; Eaton and Ensign, 1980). Both the calcium-binding protein CabC (Wang *et al.*, 2008) and cyclic adenosine monophosphate (cAMP) (Süsstrunk *et al.*, 1998; Derouaux *et al.*, 2004; Piette *et al.*, 2005) were shown to play an important role in spore germination. In addition, SsgA was shown to play a pivotal role in this process. An SsgA–GFP fusion localized at sites where germ-tubes emerged, hinting that SsgA contributes to germination-site selection (Noens *et al.*, 2007). In the absence of SsgA, less germ tubes per spore were formed, whereas controlled *ssgA* overexpression had the opposite effect. We here also showed a role for NepA in spore germination. In fact, experimental evidence shows that this protein is involved in spore dormancy. Spores of the Δ *nepA* mutant not only displayed accelerated germination on solid media but also germinated in water. The influx of water is important for the germination of the Δ *nepA* spores, as they did not germinate in the air as opposed to, for example, *mreB* mutant spores (Mazza *et al.*, 2006) and swelled normally.

Why should NepA prevent germination in water and synchronized germination? Spores should only germinate when conditions are suitable to support vegetative growth. For instance, freshly formed spores should not germinate when exposed to dew or rain, which would result in clumps of spores and hyphae that fail to be dispersed by wind to nutrient-rich environments. By introducing heterogeneity in the germination process, as occurs in the wild-type strain, bacteria deploy an effective spread-of-risk strategy. This system is an important strategy by which bacteria maximize survival (Veening *et al.*, 2008). How NepA actually prevents spore germination and introduces heterogeneity is under current investigation.

Experimental procedures

Bacterial strains and plasmids

Streptomyces coelicolor strains M145 (Kieser *et al.*, 2000), Δ *rdlAB* (Claessen *et al.*, 2004), *K106* (Δ *nepA*; Dalton *et al.*, 2007), *whiA* (Ainsa *et al.*, 2000), *whiH* (Haydon and Guest, 1991; Ryding *et al.*, 1998), Δ *chpABCDEH* (Claessen *et al.*, 2003) and Δ *chpABCDEFGH* (Claessen *et al.*, 2004) were used in this study. Cloning was performed in *Escherichia coli* Top10 or BW25113 (Datsenko and Wanner, 2000). *E. coli* ET12567 carrying plasmid pUZ8002 was used for conjugation to *S. coelicolor*. Vectors and constructs used in this work are listed in Table 1.

Table 1. Vectors and constructs used in this study.

Plasmid	Description	Reference
pGEM-T	<i>E. coli</i> vector used for cloning of PCR fragments	Promega
pGEM- <i>nepA</i>	pGEM-T containing a 280 bp internal fragment of <i>nepA</i>	This work
pIJ8630	<i>Streptomyces</i> integrative vector containing the <i>eGFP</i> gene with adapted codon usage for <i>Streptomyces</i>	Sun <i>et al.</i> (1999)
pIJ8630- <i>nepA</i>	pIJ8630 containing the 365 bp promoter region of <i>nepA</i> , transcriptionally fused to <i>eGFP</i> by using a 3' NdeI restriction site	This work
pIJ82	pSET152 derivative (Bierman <i>et al.</i> , 1992) in which a 751 bp SacI fragment of the <i>aac(3)IV</i> gene was replaced by the <i>hyg</i> gene conferring hygromycin resistance	Dr B. Gust
pIJ82- <i>nepA</i>	pIJ82 containing a 3812 bp EcoRV fragment carrying the <i>nepA</i> gene, which is derived from cosmid 2St10A7 (Redenbach <i>et al.</i> , 1996)	This work

Media and growth conditions

Streptomyces coelicolor strains were grown at 30°C on solid MS medium (Kieser *et al.*, 2000), solid SF-glucose medium (Dalton *et al.*, 2007), R5 agar medium (Kieser *et al.*, 2000), or on minimal agar medium with glucose or mannitol as the carbon source (Kieser *et al.*, 2000). For shaken liquid cultures, *Streptomyces* strains were grown at 200 r.p.m. in YEME medium (Kieser *et al.*, 2000). *E. coli* strains were routinely grown at 30°C or 37°C in Luria–Bertani (LB) medium supplemented with the appropriate antibiotics, if necessary.

Molecular techniques and cloning procedures

Cloning procedures were performed according to Sambrook *et al.* (1989). PCR reactions were performed under standard conditions in the presence of 5% DMSO. Chromosomal DNA of *S. coelicolor* was isolated as described by Verhasselt *et al.* (1989) with modifications according to Nagy *et al.* (1995). For RNA isolation, strains were grown on cellophane discs overlaying R5 agar plates. Mycelium was collected, treated with RNeasy Protect Bacteria Reagent (Qiagen) for 5 min, incubated with lysozyme (15 mg ml⁻¹) for 15 min and disrupted by sonication (six cycles of 30 s with 20 s intervals). Total RNA was extracted using the RNeasy Protect Bacteria Midi Kit (Promega) according to instructions of the manufacturer with exception that 4 ml of RLT buffer and 2.8 ml of ethanol were used. Southern and Northern hybridizations (Church and Gilbert, 1984) were performed at 63°C using a 280 bp radioactively labelled internal fragment of *nepA*, amplified with primers *nepA*-fw and *nepA*-rev (Table 2). A probe directed against 16S rDNA was used as a loading control. Probes were synthesized using the Prime-a-Gene Labelling kit

(Promega). Blots hybridized with the *nepA* and 16S rDNA probes were exposed for 4 h and 2 min respectively.

DNA microarray analysis

Total RNA was isolated from the wild-type M145 and the corresponding Δ *chpABCDEF*GH strain that had been grown for 12, 24, 36, 48, 60 and 72 h on R5 medium overlaid with cellophane discs. Labelling of genomic DNA and cDNA derived from the RNA, as well as microarray hybridizations and spot quantification were as described previously (Hesketh *et al.*, 2007). The microarrays used were spotted PCR products derived from *S. coelicolor* M145 genomic DNA (detailed at http://www.surrey.ac.uk/SBMS/Fgenomics/Microarrays/html_code/PCR_design.html). Three biological replicate time series were analysed for each strain. Genes differentially expressed between the wild type and the mutant strain were identified using the sensitive and robust Rank Products analysis technique (Breitling *et al.*, 2004; Hong *et al.*, 2006) as described by Hesketh *et al.* (2007). Genes with a probability of false prediction (pfp) of < 0.1 were considered significant. Colour maps of differentially expressed genes were generated using GeneSpring GX (Agilent Technologies).

Construction and complementation of the *nepA* mutant

The Redirect procedure (Gust *et al.*, 2002; 2003) was employed to delete the *nepA* gene. Briefly, the apramycin resistance cassette was amplified using primers *del-nepA*-fw and *del-nepA*-rev (Table 2). The resulting PCR product was used to target cosmid 2St10A7 in *E. coli* BW25113, thereby

Table 2. Primers used in this study.

<i>nepA</i> -fw	ATGGCAAGCATCCGTACC
<i>nepA</i> -rev	GGTTGATCGACGTGAAGG
<i>nepA</i> -fw-2	GGGAAGAAGGGAAGCAAAGG
<i>nepA</i> -rev-2	ATGAAGCACACCCGCACAAG
<i>PnepAGFP</i> -fw	CTCTCTAGAGGGTCTATGTTCTGACGTAT
<i>PnepAGFP</i> -rev	TTTCATATGGTTCCTCCAGAACAAGTGCG
<i>del-nepA</i> -fw	CTGGAAGCCGGTGCGCACTTGTCTGGAGGAACAACATGATTCGGGGATCCGTCGACC
<i>del-nepA</i> -rev	CGGCGCGGCAGCGCACCCGCGCCGGGCCCCCTCAGCTCATGTAGGCTGGAGCTGCTTC

Restriction sites are underlined.

replacing the entire coding sequence of *nepA*. Cosmid 2St10A7- Δ *nepA* was introduced into *S. coelicolor* by conjugation and mutants resistant to apramycin and sensitive to kanamycin were selected. Inactivation of the *nepA* gene was verified by PCR, Southern and Western analysis.

For complementation of the *nepA* mutant, a 3.8 kb EcoRV fragment of cosmid 2St10A7, carrying the *nepA* gene, was isolated and cloned into the integrative vector pIJ82 (kindly provided by Dr B. Gust). The resulting construct, pIJ82-*nepA*, was introduced into the Δ *nepA* mutant by conjugation selecting for hygromycin-resistant colonies.

Construction of plasmid pIJ8630-*nepA*

Construct pIJ8630-*nepA* was created to monitor expression of *nepA* in developing colonies. To this end, the 365 bp promoter region located upstream of *nepA* was amplified by PCR using primers *PnepAGFP-fw* and *PnepAGFP-rev* (Table 2) and cloned in the XbaI/NdeI restriction sites of pIJ8630 (Sun *et al.*, 1999). The sequence was adjusted such that the ATG of the NdeI restriction site corresponded to the ATG start codon of the *nepA* gene.

NepA extraction, gel electrophoresis and Western blotting

NepA was isolated from lyophilized *S. coelicolor* cell walls with 100% TFA (Acros organics) as described previously (Claessen *et al.*, 2002; 2003). Extracts from 5 mg cell walls were taken up in 200 μ l water and, if necessary, adjusted to pH 7 with 25% ammonia. SDS-PAGE was performed using 16% acrylamide gels prepared according to Laemmli (1970). After separation, proteins were stained with Coomassie brilliant blue G-250 (Bio-Rad), or Silver stain (Bio-Rad) or transferred to a polyvinylidene fluoride (PVDF) membrane (Roche) using semi-dry blotting. For immuno detection, polyclonal antibodies were raised in rabbits against two chemically synthesized NepA-derived peptides (Eurogentec) with sequences SGASNQSNQAQVDGSC and GVGDDNNGNSSTTQQ. Antibodies were purified from both antisera by affinity chromatography using an ACH-sepharose column. For Western blotting a mixture of the two purified NepA antibodies was used (1:2000). For signal detection, a goat anti-rabbit alkaline phosphatase conjugate (1:5000, Sigma) was used with CDP-*Star* (Roche) or NBT/BCIP as a substrate. In the former case, blots were exposed in a Lumina-ImagerF1 (Roche).

Microscopy methods

To analyse spore germination and development, strains were grown on cellophane discs placed on top of solid MS plates. Squares (2 \times 2 cm) were excised for analysis as described by Manteca *et al.* (2007). Nucleic acids were stained for 5 min with the cell-permeable, green fluorescent dye SYTO 9 and the cell-impermeable, red fluorescent dye propidium iodide (PI) to discriminate between life and death cells. For visualization of GFP, a coverslip was placed in, or on top of MS agar plates grown with recombinant *Streptomyces* strains. The wild-type strain transformed with the empty plasmid pIJ8630 served as a negative control. Samples were analysed by

confocal laser-scanning microscopy using a Leica TCS-SP-AOBS microscope or using a Zeiss Axioskop 50. Settings of the microscope were identical for all strains and adjusted such that autofluorescence observed in the negative control was negligible.

For scanning electron microscopy, cultures grown on solid MS medium were frozen in liquid nitrogen and sputter-coated with gold/palladium. Examination was performed at 5.0 kV in a JEOL field emission scanning electron microscope (type 6301F).

Spore germination and viability

Germination was quantified in a biological triplicate by inoculating spores on solid MS medium. After 2, 3, 4, 5, 6, 8, 10 and 14 h germination was assessed of at least 100 spores at each time point. Spores were considered to be germinating when one or more germ tubes were visible. To determine the total number of spores produced by the wild-type strain and the Δ *nepA* mutant, MS plates were inoculated with 1×10^7 spores. After 48 and 120 h, all spores were isolated of three plates after which a dilution series was plated to deduce the number of colony-forming units.

To test spore susceptibility, spores of the wild-type strain and the *nepA* mutant were exposed to heat (50°C in water for 60 min), sonication (up to 10 cycles of 20 s with an amplitude of 10 μ m), SDS (exposure to 2% and 10% for 4 h) and lysozyme (incubation with 10 mg ml⁻¹ up to 4 h). Spores were plated after treatment and the number of colony-forming units was determined.

To determine whether NepA is released during germination, 1×10^8 spores were taken up in 0.05 M TES (pH 8), heat-shocked for 10 min at 50°C and supplemented with the same volume of double-strength germination medium (Kieser *et al.*, 2000). Spores were incubated at 30°C while shaking at 200 r.p.m., and samples were taken after 0, 20, 40, 80, 160 and 320 min of incubation. Each sample was examined for spore germination using light microscopy. Spores and medium were separated by centrifugation, lyophilized, extracted with TFA and taken up in SDS sample-buffer prior to Western analysis.

Zeta potential distributions measurements

To determine the overall surface charge of the cell walls of spores, zeta potential distribution measurements were performed, essentially as described by van Merode *et al.* (2006). A total of 10^8 spores per ml were taken up in 10 mM K₂HPO₄/KH₂PO₄ buffer that was set to pH 2, 3, 4, 5, 7 and 9 respectively. The Zeta potential was determined by measuring the velocity of spores in a microelectrophoresis chamber upon application of a voltage difference of 150 V. Measurements were performed on a Lazer Zee Meter 501 (PenKem, Bedford Hills).

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Supporting information

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